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Article

Diurnal Regulation of MTP and Plasma Triglyceride by CLOCK Is Mediated by SHP

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SUMMARY

We examined the role of clock genes in the diurnal regulation of plasma triglyceride-rich apolipoprotein B-lipoproteins and their biosynthetic chaperone, microsomal triglyceride transfer protein (MTP). *Clock*$^{mt/mt}$ mice showed sustained hypertriglyceridemia and high MTP expression. CLOCK knockdown activated MTP promoter and reduced small heterodimer partner (*SHP*, NROB2). CLOCK upregulated SHP by binding to its E box. SHP suppressed MTP expression by binding to the HNF4α/LRH-1 at the MTP promoter. Cyclic expression of MTP after serum shock was abrogated by siCLOCK and siSHP. Plasma triglyceride and MTP showed reduced diurnal variations in *Shp*$^{-/-}$ mice. Whereas peaks and nadirs in SHP expression were inversely correlated with those of MTP, these changes were reduced in *Clock*$^{mt/mt}$ mice. Expression of Shp abrogated hypertriglyceridemia in *Clock*$^{mt/mt}$ mice. Together, these studies describe a role of Clock/Shp in the diurnal regulation of MTP and plasma triglyceride and indicate that disruptions in circadian regulation might cause hyperlipidemia.

INTRODUCTION

Several biological, physiological, and behavioral activities show a characteristic recurrence with 24 hr intervals attuned to sunrise and sunset. Light entrains central clocks present as two lateral suprachiasmatic nuclei in the hypothalamus via the retinohypothalamic tract. The circadian clock arises from autoregulatory transcriptional, translational, and posttranslational feedback loops of few transcription factors known as clock genes, including circadian locomotor output cycles kaput (*Clock*), brain and muscle aryl hydrocarbon receptor nuclear translocator-like 1 (*Bmal1*), neuronal PAS-containing protein 2 (*NPAS2*), period genes (*Per1*, *Per2*, and *Per3)*, and cryptochrome genes (*Cry1* and *Cry2*) (Dunlap, 1999; Reppert and Weaver, 2001; Lowrey and Takahashi, 2004).

Clock/Bmal1 or Npas2/Bmal1 heterodimers bind E box sequences present in the promoter regions of *Per1/2/3* and *Cry1/2* genes and enhance their expression, constituting a positive loop (Gekakis et al., 1998). When concentrations of Per and Cry proteins increase, they form heterodimers and suppress transcription of *Bmal1*, forming a negative feedback loop (Darlington et al., 1998). Besides this circuitry, circadian clock signals are transmitted to other clock-controlled genes via different transcription factors, leading to circadian variations in various biological and behavioral activities. For example, Clock/Bmal1 enhance expression of Rev-erbα and albumin D element-binding protein (DBP) that regulate bile acid and drug metabolisms (Green et al., 2008; Lowrey and Takahashi, 2004; Hussain and Pan, 2009). Rev-erbα regulates bile acid metabolism by regulating CYP7A1 via regulation of repressors Shp or E4bp4 or by an indirect mechanism involving Insig2 (Duez et al., 2008; Le Martelot et al., 2009). Similarly, DBP is upregulated by the binding of Clock/Bmal1 to its E box sequences (Ripperger et al., 2000). DBP and its homologs play an important role in drug detoxification (Gachon et al., 2006). In addition to being a clock-controlled gene, Rev-erbα also downregulates Bmal1 and therefore is also a component of the central clock genes (Preitner et al., 2002). In contrast to the suppression of Bmal1 by Rev-erbα, ROR transcription factors upregulate Bmal1; therefore, Rev-erbα/ROR/Bmal1 also constitute an additional regulatory loop that stabilizes the central circadian loops of Clock/Bmal1/NPAS2 and Crys/Pers (Ueda et al., 2002; Gachon et al., 2006; Liu et al., 2008).

In addition to suprachiasmatic nuclei, clock and clock-controlled genes are expressed in other tissues (Sakamoto et al., 1998; Yamamoto et al., 2004; Lowrey and Takahashi, 2004). Moreover, rhythmic expression of these genes can be induced in cultured cells after brief exposure to high serum concentrations (Balsalobre et al., 1998). Thus, several cells are capable of expressing self-sustained circadian rhythms after appropriate entrainment. It is believed that the master clock synchronizes peripheral clocks involving neural connections and humoral chemicals.

The role of *Clock* is studied using *Clock* mutant mice (*Clock*$^{mt/mt}$) that express a dominant-negative protein (Vitaterna et al., 1994; King et al., 1997). *Clock*$^{mt/mt}$ are arrhythmic, exhibiting longer circadian periods and decreased amplitude of locomotors activity (Vitaterna et al., 1994). These mice show several physiologic abnormalities, such as reduced fertility (Miller et al., 2004), obesity, and the metabolic syndrome (Turek et al., 2005). It is unclear how expression of a mutant Clock protein causes these metabolic abnormalities. Moreover, polymorphisms in *CLOCK* gene are associated with metabolic syndrome and obesity in humans (Garaulet and Madrid, 2009), but molecular mechanisms

have not been elucidated. Our aim was to find out how CLOCK regulates plasma lipids to gain insights into hyperlipidemia that might contribute to metabolic syndrome and obesity.

Several studies have shown that plasma triglyceride show diurnal variations (Seaman et al., 1965; Schlierf and Dorow, 1973; Rudic et al., 2004; Pan and Hussain, 2007; Pan and Hussain, 2009). Plasma triglyceride show persistent diurnal rhythmicity in rats fasted for 96 hr (Escobar et al., 1998). The extent of the increases in triglyceride were, however, lower than those observed in the fed state. Plasma triglycerides are carried in apolipoprotein B-lipoproteins. We showed that plasma triglyceride exhibit diurnal variations due to changes in apoB-lipoproteins (Pan and Hussain, 2007). However, little is known about the mechanisms that control diurnal changes in plasma triglyceride and apoB-lipoproteins.

Biosynthesis of apoB-lipoproteins requires a dedicated chaperone, microsomal triglyceride transfer protein (MTP), which transfers lipids in vitro between membranes. Mutations in the *MTTP* gene are associated with an absence of plasma apoB-lipoproteins in abetalipoproteinemia. MTP expression is regulated at the transcriptional level (Hussain et al., 2008). It is known that proximal 150 bp sequences in the *MTTP* promoter contain several regulatory sites (Hagan et al., 1994). The binding of HNF4α to the HNF4 site is critical for *MTTP* expression, as *Hnf4α*$^{-/-}$ mice do not express MTP (Hayhurst et al., 2001). HNF1α synergistically activates HNF4α-mediated *MTTP* expression (Sheena et al., 2005). Binding of RXR/PPARs and FOXA2 to DR1 element enhances, whereas binding of NR2F2 to the same element reduces, *MTTP* expression (Kang et al., 2003). Binding of NR2F1 to DR1 has been suggested to suppress *MTTP* expression in undifferentiated intestinal cells (Dai et al., 2010). SREBP1c decreases expression by interacting with IRE/SRE elements (Sato et al., 1999). Furthermore, different activators and repressors modulate *MTTP* expression. For example, PGC1α and PGC1β modulate *MTTP* transcription by interacting with transcription factors that bind to DR1 element (Wolfrum and Stoffel, 2006). SHP reduces MTP expression by interacting with HNF4α and LRH-1 (Hirokane et al., 2004; Huang et al., 2007).

We have shown a correlation between MTP expression and plasma lipids (Pan and Hussain, 2007). Changes in plasma lipids/lipoproteins and MTP expression are altered by restricted feeding and abolished by extended exposure to light (Pan and Hussain, 2007). We observed that *Clock* is important for calorie-restricted food entrainment of intestinal nutrient absorption and expression of different nutrient transporters (Pan and Hussain, 2009). These studies led to the hypothesis that visual cues and clock genes might play a role in the circadian regulation of MTP and plasma triglyceride. To test this hypothesis, we studied changes in plasma lipids and hepatic MTP in wild-type and *Clock*$^{mt/mt}$ mice fed ad libitum and subjected to food entrainment, and we examined mechanisms by which Clock regulates hepatic MTP and plasma triglyceride.

RESULTS

Clock Is Important for Diurnal Regulation of Plasma Triglyceride and Hepatic Mtp

Clock is a critical transcription factor controlling circadian rhythms. To evaluate the role of Clock in regulating plasma lipids and hepatic MTP, we used *Clock*$^{mt/mt}$ (mutant) and their wild-type (*Clock*$^{wt/wt}$) siblings. Clock protein and mRNA were high just before the lights went on (Figure S1A available online). *Clock*$^{mt/mt}$ mice expressed low levels of Clock that did not show circadian variations seen in wild-type mice (Figure S1B). Similarly, expression of several other clock genes, including Npas2, Bmal1, Rev-erbα, Dbp, and Pers, was repressed in mutant mice (Figures S1C–S1L). Plasma triglycerides in *Clock*$^{wt/wt}$ were 2- to 3-fold higher at 24 hr compared to 12 hr (Figure 1A), owing to changes in apoB-lipoproteins (Figure 1B). In *Clock*$^{mt/mt}$, plasma triglyceride (Figure 1B) did not change. These mice had higher plasma triglyceride at all times seen normally at night in wild-type mice due to the absence of nadirs in the day. In contrast to triglyceride, changes in plasma and apoB-lipoprotein cholesterol were less prominent (Figures 1C and 1D). MTP activity (Figure 1E), mRNA (Figure 1F), and protein (Figure 1G) showed diurnal variations in the *Clock*$^{wt/wt}$, but not in *Clock*$^{mt/mt}$ livers. Overall, these data indicate that Clock is important for daily regulation of plasma triglyceride and hepatic Mtp.

Clock Is Required for Optimal Food Entrainment of Plasma Triglyceride and Hepatic Mtp

To understand mechanisms behind high plasma triglyceride in mutant mice, we studied food intake, hepatic lipoprotein assembly, and response to food entrainment. *Clock*$^{mt/mt}$ mice consumed more food (Figure S2A) and synthesized and secreted more triglyceride-containing lipoproteins (Figure S2B). To examine whether shifting mice from unrestricted feeding to daytime scheduled feeding alters daily changes in plasma lipids, mice were provided food between 11 AM and 3 PM for 10 days. In *Clock*$^{wt/wt}$ mice, plasma triglyceride (Figure 2A) and cholesterol (Figure 2C) were significantly higher at the time of food availability due to increases in apoB-lipoproteins (Figures 2B and 2D). The food entrainment response was significantly reduced in mutant mice (Figures 2A–2D). Expression of hepatic MTP activity (Figure 2E), mRNA (Figure 2F), and protein (Figure 2G) was increased at the time of food availability, and these altered expressions were significantly dampened in mutant mice. All of the clock genes did respond to food entrainment, and their mRNA levels either increased or decreased at the time of food availability in wild-type mice (Figures S2C–S2L). Clock genes did not respond to or responded poorly to food entrainment in mutant mice. These studies indicate that food entrainment enhances plasma triglyceride and hepatic Mtp at the time of food availability and that the optimum responses require normal Clock expression. Therefore, both food availability and Clock are important modifiers of MTP expression.

Clock Suppresses MTP Expression in Hepatoma Cells

The above studies in *Clock*$^{mt/mt}$ could represent direct regulation of *Mttp* by Clock, or they might represent an accommodation to the long-term expression of the dominant-negative Clock protein. To examine the role of CLOCK in MTP regulation in human cell lines, we reduced its expression using RNAi in Huh-7 cells. siCLOCK significantly reduced *CLOCK* mRNA and protein, increased *MTTP* mRNA protein and activity, and had no effect on *GAPDH* mRNA and protein (Figures 3A and 3B)

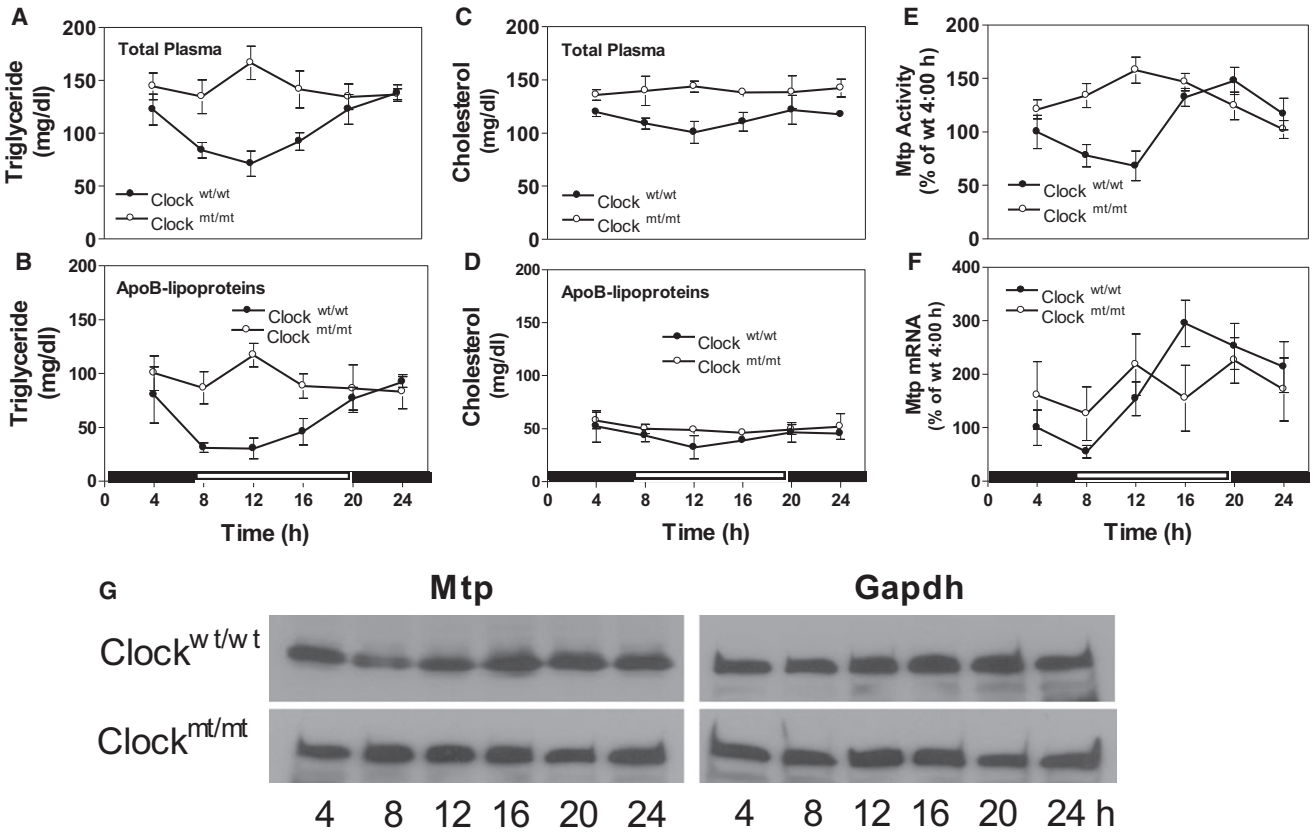

Figure 1. Changes in Plasma Lipids and Hepatic MTP in Mice Fed Chow Ad Libitum

(A–G) *Clock*$^{mt/mt}$ and their wild-type siblings (*Clock*$^{wt/wt}$) were kept in 12 hr light-dark cycle and fed chow at ad libitum. Triglyceride and cholesterol were measured in total plasma (A and C) and apoB-lipoproteins (B and D). Liver samples were used to measured MTP activity (E), mRNA (F), and protein (G). Each time represents mean ± SD; n = 6. Western blot is representative of n = 3.

compared with siCONTROL, indicating that CLOCK is a negative regulator of *MTTP* expression.

To explore the role of other clock genes, these cells were exposed to siRNAs against different clock genes (Figures 3C and 3D). Not only *CLOCK*, but also *BMAL1* and *NPAS2*, negatively regulated *MTTP* (Figures 3C and 3D). On the other hand, DBP, PER2, and CRY1 had no effect on MTP expression, whereas REV-ERBα, PER1+CRY1, and PER2+CRY2 positively regulated *MTTP* expression. These studies show that the positive loop of the circadian clock negatively regulates MTP expression.

Experiments were then performed to determine whether *MTTP* induction involves transcriptional and/or translational mechanisms. Cycloheximide, a protein translation inhibitor, and actinomycin D, an mRNA transcription inhibitor, decreased MTP activity with time (Figure 3E) in both siCONTROL- and siCLOCK-treated cells. *MTTP* mRNA remained elevated in cycloheximide-supplemented siCLOCK-treated cells, indicating that CLOCK may not require protein synthesis for the transcriptional regulation of *MTTP* (Figure 3F). As expected, actinomycin D reduced MTP mRNA with time in siCONTROL cells. In siCLOCK-treated cells, MTP mRNA levels were not elevated in the presence of actinomycin D, suggesting that CLOCK might control *MTTP* expression involving transcriptional mechanisms.

CLOCK Regulates MTP Using SHP

Attempts were made to determine how CLOCK suppresses *MTTP* transcription. Analysis of the *MTTP* promoter did not reveal any E box sequences; therefore, we hypothesized that CLOCK might regulate expression of critical transcription factor(s) that control MTP expression (Hussain et al., 2008; Dai et al., 2010). Increased expression of activators or decreased expression of repressors in siCLOCK-treated cells could explain higher MTP levels. siCLOCK increased MTP mRNA (Figure 4A) and had no effect on RXRα (Figure 4A) but decreased PPARα, PGC-1α, HNF4α, and PGC-1β expression (Figure 4A). As these activators were not changed or were reduced, we concluded that CLOCK might not regulate MTP by upregulating activators. Therefore, we studied changes in *MTTP* repressors. siCLOCK reduced SHP mRNA (Figure 4A) and protein (Figure 4B) but had no effect on NR2F2 mRNA (Figure 4A), indicating that CLOCK might enhance *MTTP* expression by suppressing SHP. CLOCK has been shown to bind to the E box sequences present in the *SHP* promoter and regulate its expression (Oiwa et al., 2007). We confirmed this by performing ChIP using anti-Clock antibodies (Figure 4C, siCONTROL). We detected significantly less CLOCK binding to SHP E box after siCLOCK treatment compared with siCONTROL, implying that CLOCK may directly

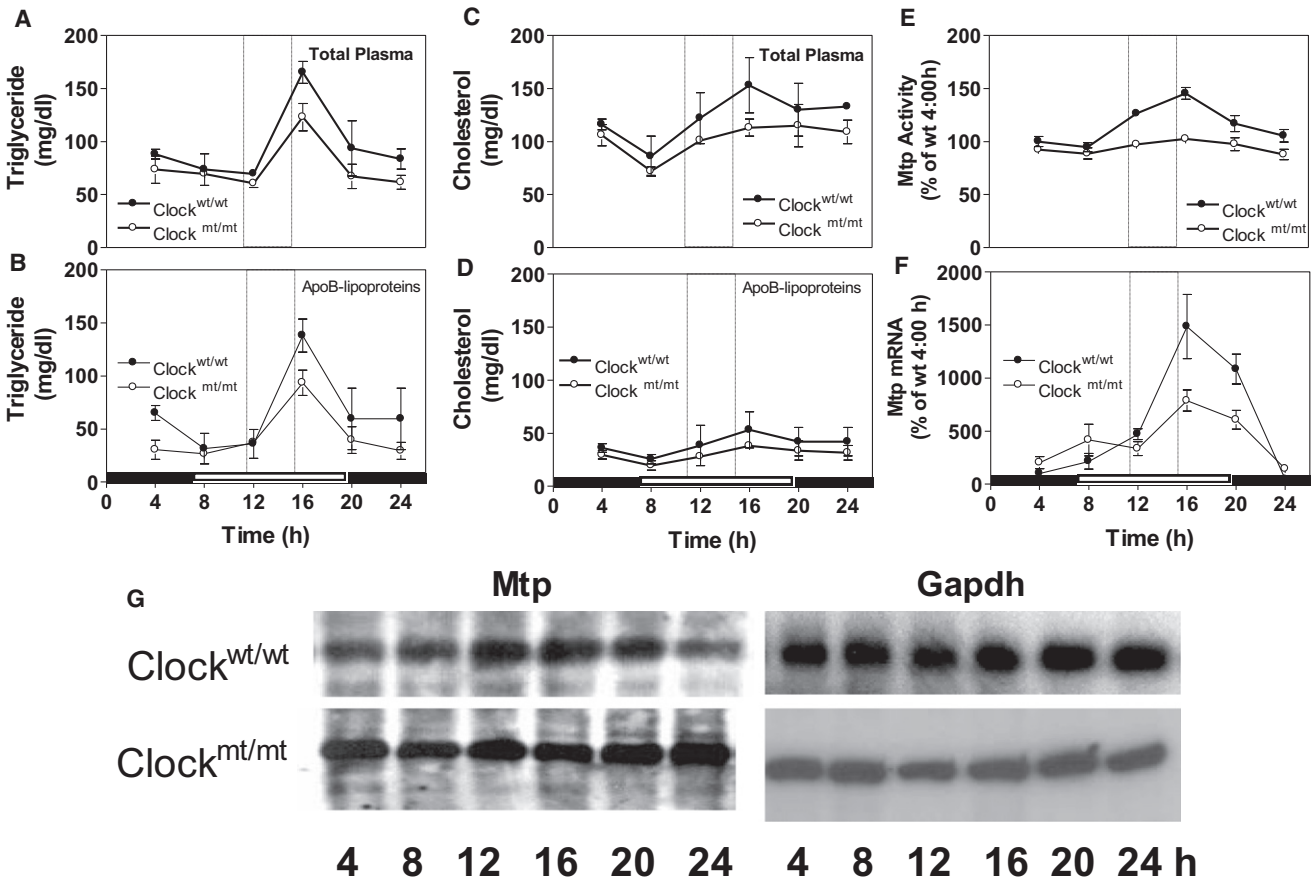

Figure 2. Changes in Plasma Lipids and Hepatic MTP in Mice Subjected to Food Restriction

(A–G) *Clock*$^{mt/mt}$ and *Clock*$^{wt/wt}$ siblings were kept in 12 hr light-dark cycle and fed chow from 11 AM to 3 PM for 10 days. Triglyceride and cholesterol were measured in total plasma (A and C) and apoB-lipoproteins (B and D). Liver samples were used to measure MTP activity (E), mRNA (F), and protein (G). Each time point represents mean ± SD; n = 6. Western blotting representative of n = 3.

upregulate SHP. Therefore, SHP might be a clock-controlled gene responsible for Clock-mediated MTP regulation.

To evaluate the role of SHP in MTP regulation, Huh-7 cells were treated with siSHP. This treatment significantly reduced SHP mRNA (Figure 4D) and protein (Figure 4E) but increased MTP mRNA (Figure 4D), protein (Figure 4E), and activity (Figure 4F). siGAPDH had no effect on MTP and SHP mRNA (Figure 4D). To establish further a relation between SHP and MTP, we expressed SHP using adenoviruses or plasmids. Expression of SHP reduced MTP activity, mRNA, and protein (Figures 4G–4I). These studies indicate that SHP represses MTP expression, confirming other studies (Hirokane et al., 2004; Huang et al., 2007).

Attempts were then made to understand how SHP suppresses *MTTP* expression. SHP has been shown to reduce *MTTP* expression by binding to HNF-4α (Hirokane et al., 2004) and LRH-1 (Huang et al., 2007). Therefore, we studied the binding of SHP to HNF4α, LRH-1, and HNF-1α transcription factors associated with their putative binding sites on the *MTTP* promoter. Under normal conditions (siCONTROL), anti-SHP antibodies precipitated sequences occupied by HNF4α, LRH-1, and HNF1α (Figure 4J). The binding of SHP to the HNF4α-binding site of the *MTTP* promoter was significantly reduced in siCLOCK- and siSHP-treated cells (Figure 4J). Compared to HNF4α, the binding of SHP to LRH-1 and HNF1 sites was decreased to a lesser extent in siCLOCK-treated cells. These studies indicate that knockdown of CLOCK and SHP significantly decreases the binding of SHP to the *MTTP* promoter. To substantiate the effect of SHP on *MTTP* promoter, we used constructs that express luciferase under the control of a basal *MTTP* promoter (Dai et al., 2010). Cells were cotransfected with pMTP-205-Luc (expresses firefly luciferase under the control of 205 bp *MTTP* promoter), *Renilla* luciferase (a transfection control), and plasmids expressing either GFP or SHP (Figure 4K). SHP expression reduced basal MTP promoter activity by 50%. Conversely, cotransfection of pMTP-204 with siSHP or siSHP/siCLOCK increased promoter activity by ~4-fold (Figure 4L). These studies indicate that SHP might suppress *MTTP* expression by binding to HNF4α and LRH-1. We posit that CLOCK increases SHP, and enhanced SHP expression leads to decreased MTP expression.

Cyclic Expression of MTP Is Regulated by CLOCK and SHP

The above studies indicate that CLOCK regulates MTP expression by modulating SHP. However, these studies do not affirm

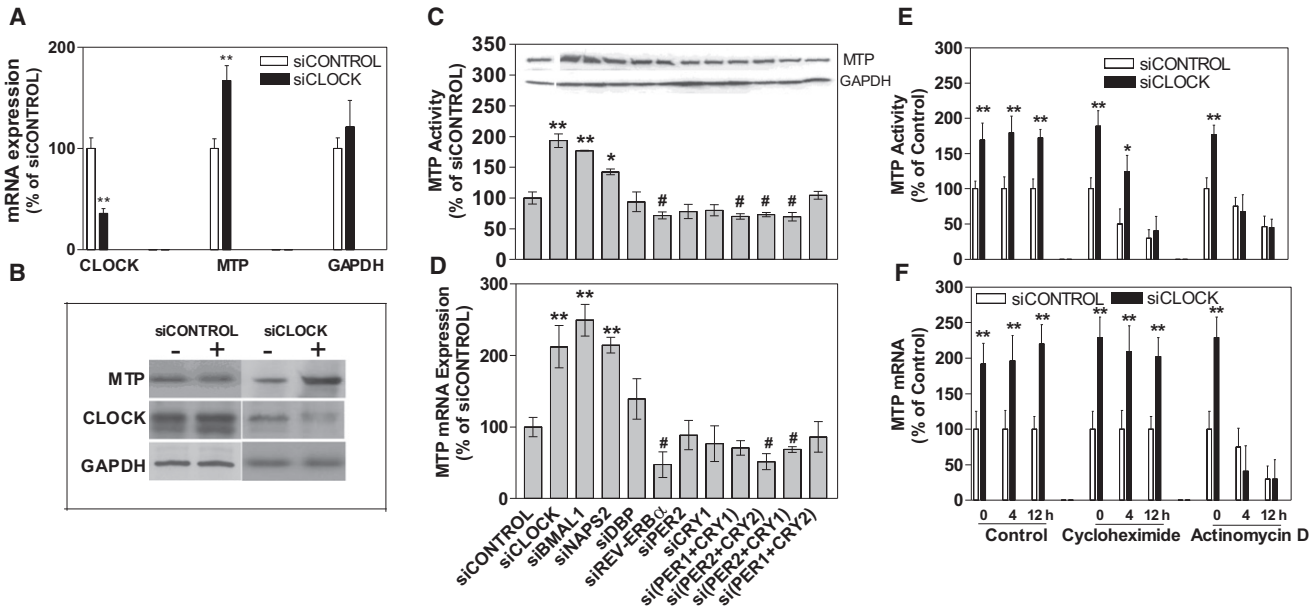

Figure 3. Clock Knockdown Increases MTP Expression

(A and B) Huh-7 cells (n = 3) were treated with siCLOCK or siCONTROL for 72 hr. *CLOCK*, *MTTP*, and *GAPDH* mRNA (A) and protein (B) were determined by qRT-PCR and immunoblotting, respectively. Changes in mRNA with respect to siCONTROL-treated cells are presented. **p < 0.05, compared to siCONTROL.

(C and D) Huh-7 cells were treated with different siRNA for 72 hr. Cells were used to measure triglyceride transfer activity (C), protein (inset), and mRNA (D) levels of MTP.

(E and F) Huh-7 cells (n = 3) were treated with siCLOCK or siCONTROL for 72 hr and treated with either cycloheximide (20 μM) or actinomycin D (2 μM) for 4 or 12 hr and were used to measure MTP activity (E) and mRNA (F). *p < 0.5 and **p < 0.05 compared to siCONTROL.

whether these genes play a role in the rhythmic expression of MTP. It is known that brief exposure of cells to 50% horse serum induces circadian expression of various genes (Balsalobre et al., 1998). Therefore, HepG2 (Figure S3) and Huh-7 (Figure 5) cells were incubated in serum-free media for 18 hr, exposed to 50% serum for 2 hr, washed, and incubated in serum-free media. MTP mRNA and activity were augmented immediately after serum supplementation. Subsequently, it showed rhythmic expression (Figures 5A and 5B). SHP mRNA (Figure 5C) also showed cyclic expression after 50% serum exposure; however, it was anti-phasic with MTP. First, there was no induction in SHP expression soon after serum shock. Second, peak expression of SHP occurred at times of low MTP expression. GAPDH mRNA did not change after serum shock and did not exhibit cyclic expression (Figure 5C). These studies show that serum shock induces cyclic expression of MTP and SHP. Their expression exhibits disparate peaks and nadirs.

Next, we explored the role of *CLOCK* and *SHP* in the cyclic regulation of *MTTP* mRNA by reducing their expression and then subjecting them to serum shock. Cells treated with siCONTROL showed high MTP expression soon after serum supplementation and a subsequent peak at 32–36 hr (Figure 5D). Expression patterns of MTP and SHP showed slightly different temporal expression than seen in Figure 5A, probably because these cells were subjected to transfections. CLOCK itself did not show significant rhythmic expression and is in concert with other studies (Balsalobre et al., 1998). Instead, its association with Bmal1 and transport to the nuclei show rhythmic changes (Tamaru et al., 2003; Gallego and Virshup, 2007). Again, GAPDH was resilient to serum shock, and its expression did not change with time. siCLOCK (Figure 5E) enhanced MTP expression (compare with Figure 5D) after serum shock and abrogated its subsequent rhythmic expression. siCLOCK reduced SHP as before (data not shown) but abolished rhythmic changes in SHP mRNA (Figure 5E) and protein (Figure 5G), indicating that CLOCK is needed for the cyclic expression of SHP after serum shock. Enhanced expression of MTP after serum supplementation might be secondary to reduced SHP expression. siCLOCK reduced CLOCK (data not shown), but the residual mRNA did not change significantly throughout the experiment compared to 0 time point. Similar to siCLOCK, siSHP also enhanced MTP expression soon after serum supplementation (Figure 5F). However, siSHP-treated cells failed to exhibit cyclic expression of *MTTP*. Cosinar analysis compared cyclic changes over 48 hr and indicated a period of 23.7 ± 0.55 and 25.2 ± 1.07 hr for Mtp and Shp mRNA, respectively (Figures 5D–5F). Comparison of changes in two successive 24 hr time intervals gave a p value of 0.003 for both Mtp and Shp, indicating significant cyclic changes. Similar analyses in siShp- and siClock-treated cells gave a p value of 0.274 and 0.198, indicating loss of cyclic change in Mtp expression. These studies indicate that MTP exhibits a complex response to serum shock involving early upregulation followed by rhythmic expression. The first immediate response is enhanced when CLOCK and SHP expressions are reduced, indicating that these proteins dampen serum response. However, these proteins are required for the subsequent cyclic expression of MTP. To gain biochemical insights into the rhythmic expression of MTP, we studied the temporal

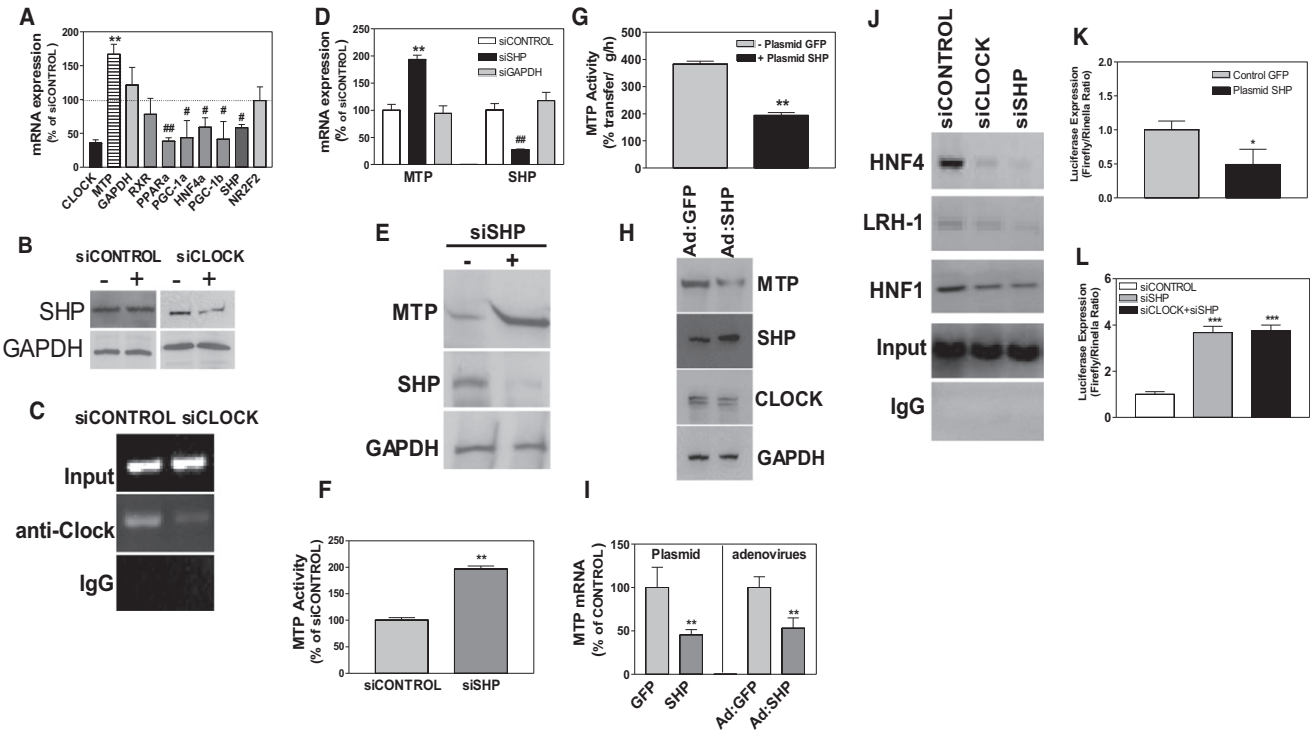

Figure 4. SHP Suppresses MTP Expression

(A–C) Huh-7 cells were transfected with siCLOCK or siCONTROL. After 72 hr, mRNA levels of activators and suppressors of *MTTP* expression were quantified by qRT-PCR (A). Data represent percentage of mRNA in siCLOCK-treated cells compared with siCONTROL. SHP and GAPDH protein were detected by immunoblotting (B). Cells were subjected to ChIP using anti-Clock antibodies, and immunoprecipitates were used to amplify E box sequences in the *SHP* promoter (C). Data are representative of n = 3. $^{\#}p < 0.5$, $^{\#\#}p < 0.01$, and $^{**}p < 0.05$ compared to siCONTROL.

(D) Huh-7 cells were treated with or without siGL2 (siCONTROL), siSHP, or siGAPDH for 72 hr and were used to measure MTP and SHP mRNA. $^{**}p < 0.05$ compared to siCONTROL.

(E and F) Huh-7 cells were treated with siSHP for 72 hr and used to measure MTP, SHP, and GAPDH protein by immunoblotting (E) and MTP activity (F). $^{**}p < 0.05$ compared to siCONTROL.

(G) Huh-7 cells were transfected with plasmids expressing human SHP (+Plasmid SHP) or not (−Plasmid GFP). MTP activity was measured after 72 hr. $^{**}p < 0.05$.

(H) Huh-7 cells were infected with adenoviruses expressing green fluorescent protein (Ad:GFP) or mouse SHP (Ad:SHP). After 48 hr, cells were used to detect MTP, SHP, CLOCK, and GAPDH proteins.

(I) Cells were transfected with indicated plasmids or infected with viruses. After 72 hr, MTP mRNA were quantified by qRT-PCR. $^{**}p < 0.05$.

(J) Binding of SHP to different *cis* elements in the MTTP promoter. Cells were treated with siCONTROL, siCLOCK, or siSHP. After 72 hr, cells were subjected to ChIP using anti-SHP antibodies. Immunoprecipitates were used to amplify sequences corresponding to HNF4, LRH-1, and HNF1 *cis* elements.

(K) pMTP-204 promoter construct expressing luciferase under the control of basal human *MTTP* promoter or pCMV-*Renilla* luciferase (control vector) were introduced in Huh-7 cells (n = 3). In addition, cells also received a control- (GFP) or SHP-expressing plasmid. Luciferase activities were measured after 72 hr. $^{*}p < 0.5$.

(L) pMTP-204 promoter construct or pCMV-*Renilla* luciferase (as a control vector) were transiently transfected in Huh-7 cells (n = 3). In addition, these cells received siCONTROL, siSHP, or siCLOCK+siSHP. Luciferase activity was measured after 48 hr. $^{***}p < 0.001$ compared to siCONTROL.

binding of two major activators, HNF4α and HNF1α, to the *MTTP* promoter. The binding of HNF4α and HNF1α to the *MTTP* promoter was low at 16 hr and high at 36 hr (Figure 5H). In siCLOCK- and siSHP-treated cells, binding of these transcription factors was similar at two different times. These data indicate that rhythmic expression of MTP might be related to the binding of different amounts of HNF4α/HNF1α to their respective sites on the *MTTP* promoter. We also evaluated the role of SHP in the rhythmic expression of MTP (Figure 5I) by performing ChIP using anti-Shp antibodies and amplifying sequences corresponding to different *cis* elements. The binding of SHP to HNF4, HNF1, and LRH-1 sites in the *MTTP* promoter was high at 16 hr and low at 36 hr (Figure 5I, siCONTROL). These time-dependent changes were not evident in siCLOCK- and siSHP-treated cells. Based on these studies, we posit that rhythmic expression of MTP involves binding of different amounts of HNF4α and HNF1α to their respective sites and differential association of SHP at the HNF4/HNF1/LRH-1 sites in the *MTTP* promoter. At the time of high *MTTP* expression, its promoter has more HNF4α and HNF1α associated with it. At the same time, the amounts of SHP present with HNF4α and LRH-1 are low. At the time of low expression, the amounts of HNF4α and HNF1α associated with the *MTTP* promoter are low. But, at this time, there are higher amounts of SHP associated with the *MTTP* promoter.

Shp Is Important for the Circadian Regulation of Plasma Triglyceride and Hepatic *Mttp*

Our studies in Huh-7 cells indicate that SHP plays an important role in the cyclic expression of MTP. To determine whether Shp

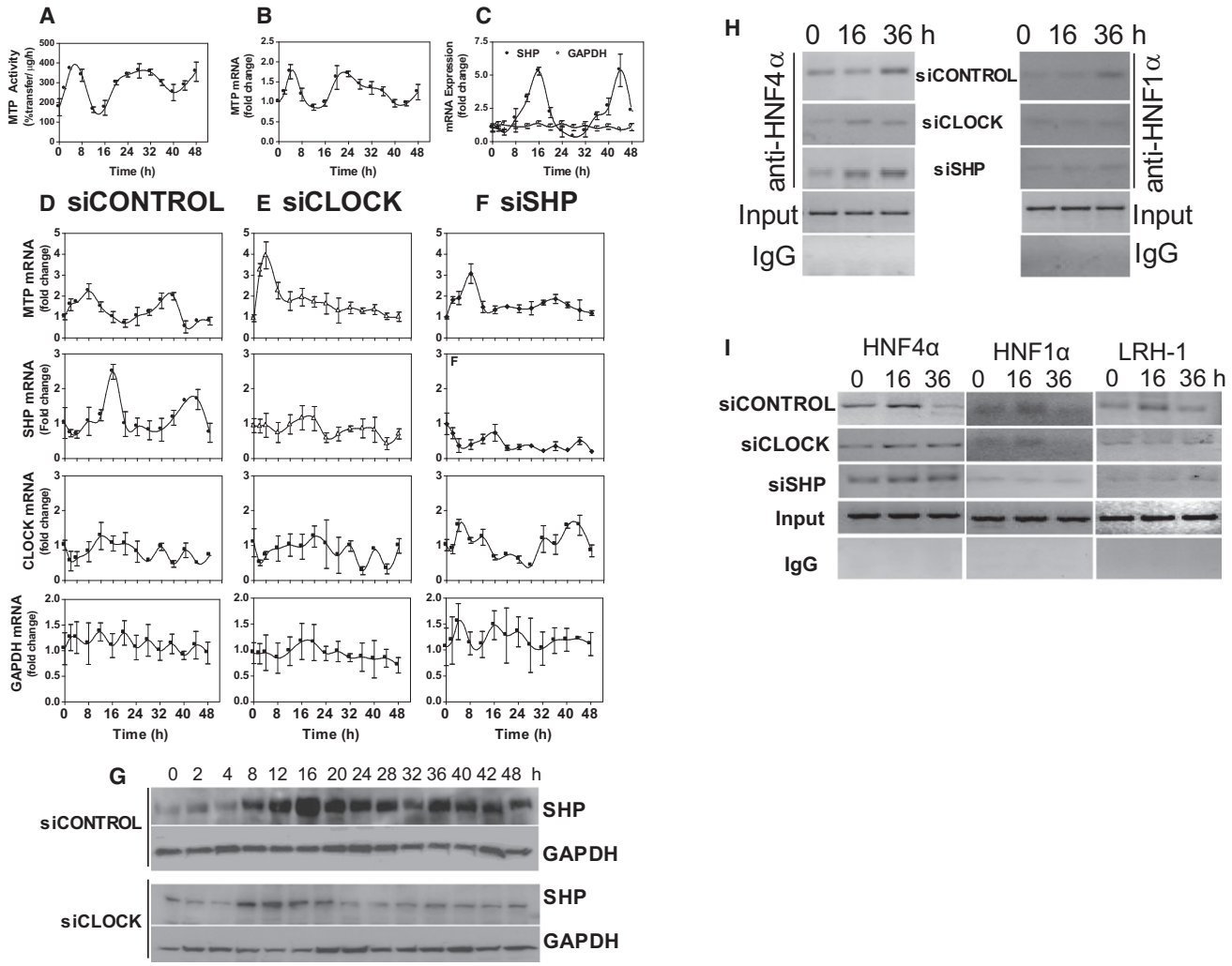

Figure 5. CLOCK and SHP Regulate Cyclic Expression of MTP in Huh-7 Cells

(A–C) Huh-7 cells were cultured in DMEM containing 10% serum. Confluent monolayers were incubated in serum-free media for 18 hr and for 2 hr in media containing 50% fetal calf serum. Cells were washed and incubated in serum-free media. At indicated times, three wells were harvested to measure MTP activity (A), mRNA (B), and SHP/GAPDH mRNA (C) in triplicate.

(D–G) Huh-7 cells were transfected with siCONTROL (D), siCLOCK (E), or siSHP (F). After 48 hr, cells were subjected to 2 hr serum shock. At indicated times, mRNA levels of different genes were measured (D–F). For comparison, mRNA levels were normalized with 18S rRNA, and the values at 0 hr were normalized to 1. Other values represent fold change compared to 0 hr. Cell lysates were also used to detect SHP and GAPDH protein (G).

(H) Huh-7 cells were treated with different siRNA and subjected to serum shock. Cells were harvested at 0, 16, and 36 hr and subjected to ChIP assays using anti-HNF4α or anti-HNF1α antibodies. Immunoprecipitates were used to amplify HNF4 or HNF1 sites in the *MTTP* promoter.

(I) Huh-7 cells were treated with siRNA and subjected to serum shock. Cells were harvested at 0, 16, and 36 hr and used for ChIP assays using anti-SHP antibodies. The extracted DNAs from the immunoprecipitates were amplified using primers specific for human HNF4, HNF1, and LRH-1 sites in the *MTTP* promoter. Data are representative of n = 3.

is essential for the circadian regulation of Mtp and plasma triglyceride in vivo, we studied their regulation in $Shp^{-/-}$ and $Shp^{+/+}$ mice (Figure 6). As before, plasma triglyceride showed diurnal variations in $Shp^{+/+}$ mice, with higher levels at night (Figure 6A) owing to changes in apoB-lipoproteins (Figure 6B). In $Shp^{-/-}$ mice, plasma triglyceride did not show significant variations within 24 hr and exhibited sustained hypertriglyceridemia (Figures 6A and 6B) similar to that seen in $Clock^{mt/mt}$ mice. Moreover, $Shp^{-/-}$ mice had high plasma apoB100 and apoB48 (Figure S4), absorbed more lipids (Figure S4B), and consumed more food (Figure S4C). Hepatic triglyceride and cholesterol levels were similar in wild-type and knockout mice (Figures S4D–S4E). In $Shp^{+/+}$ livers, MTP activity, protein, and mRNA (Figures 6C–6E) showed diurnal variations with high levels seen at night. In $Shp^{-/-}$ liver, however, Mtp did not show significant variations during 24 hr, and they remained high at all times (Figures 6C–6E). The expression profile of *Mttp* in $Shp^{-/-}$ was similar to that in $Clock^{mt/mt}$. Therefore, consideration was given to the possibility that Shp might regulate *Mttp* involving clock genes. Many clock genes exhibited similar or reduced amplitudes in $Shp^{-/-}$ mice compared with $Shp^{+/+}$ mice, except for Rev-erbα and Dbp,

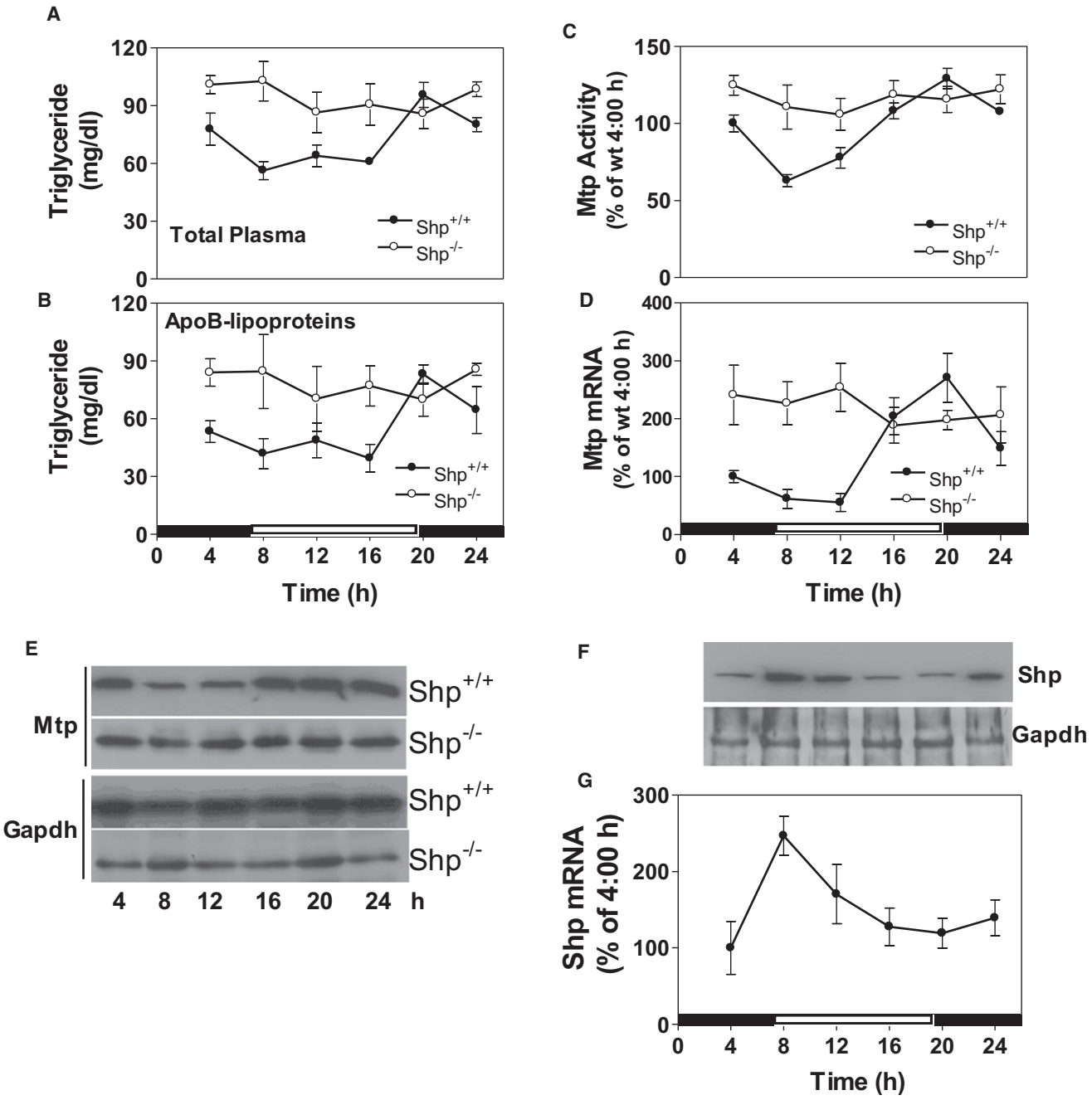

Figure 6. Plasma Triglyceride and Hepatic Mtp Do Not Change in *Shp*$^{-/-}$ Mice

(A–E) *Shp*$^{-/-}$ and control (*Shp*$^{+/+}$) mice were kept in 12 hr light-dark cycle and fed chow ad libitum. Triglycerides were measured in total (A) and apoB-lipoproteins (B). Liver samples were used to measured Mtp activity (C), mRNA (D), and protein (E). Each time point represents mean ± SD; n = 4–6.

(F and G) Livers were collected at indicated times from male C57BL/6J mice fed ad libitum and used to measure Shp and Gapdh protein (F) and Shp mRNA (G). They were plotted as line graphs and error bars, respectively. Mean ± SD; n = 6.

which showed a significant shift in their rhythmic expression (data not shown). Certainly, their expression and diurnal variations were not as dampened as in *Clock*$^{mt/mt}$ mice (Figure S1). These studies indicate that *Shp* expression is important for the diurnal regulation of plasma triglyceride and hepatic *Mttp*, but not for clock genes.

Circadian Regulation of Shp in *Clock*$^{wt/wt}$ and *Clock*$^{mt/mt}$ Siblings

The studies described so far indicated that plasma triglyceride and hepatic Mtp profiles are similar in *Shp*$^{-/-}$ and *Clock*$^{mt/mt}$ mice. Furthermore, cell culture experiments indicate that CLOCK might regulate MTP via SHP. If *SHP* is the clock-controlled gene

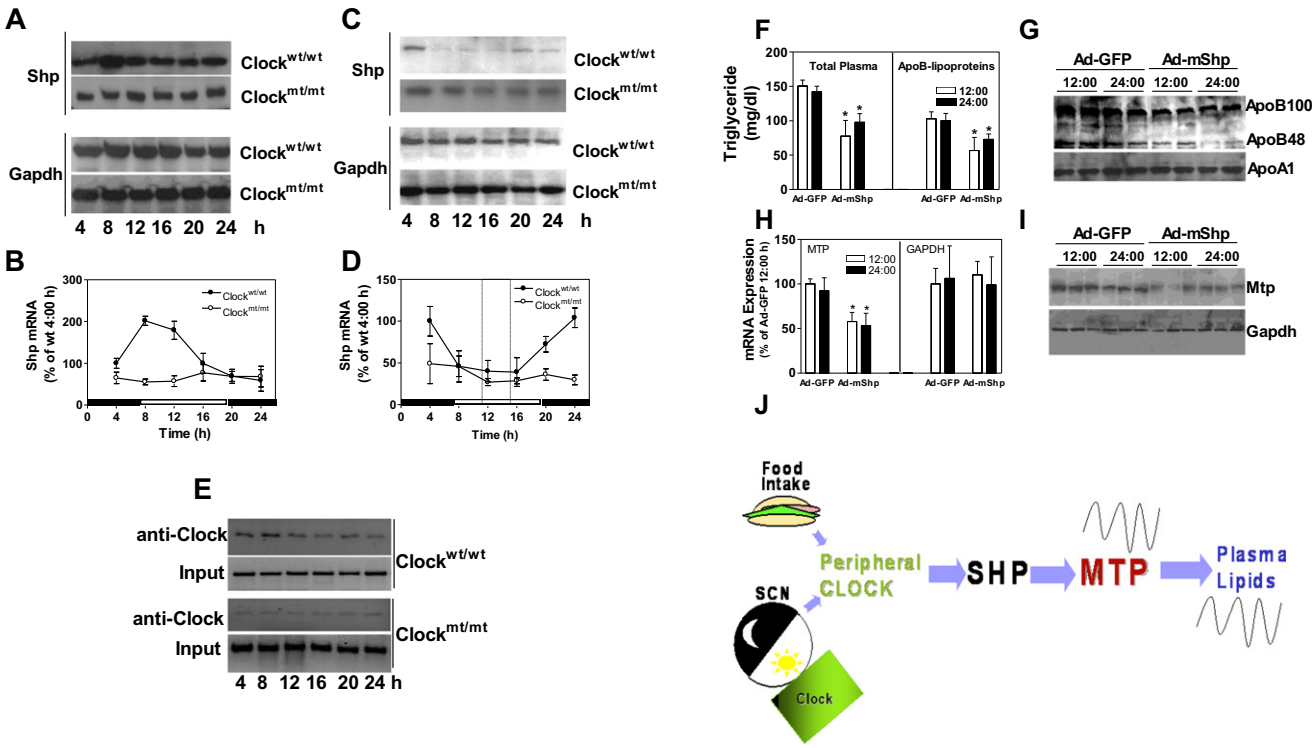

Figure 7. Circadian Regulation of *Shp* and Effect of SHP Expression in *Clock*$^{wt/wt}$ and *Clock*$^{mt/mt}$ Siblings

(A and B) Livers were collected at indicated times from male *Clock*$^{mt/mt}$ and *Clock*$^{wt/wt}$ siblings fed chow ad libitum to measure Shp/Gapdh protein (A) and *Shp* mRNA (B). Each time point for mRNA represents mean ± SD; n = 6.

(C and D) Expression of Shp/Gapdh protein (C) and mRNA (D) in male *Clock*$^{mt/mt}$ and *Clock*$^{wt/wt}$ mice kept in 12 hr light-dark cycle and fed chow from 11 AM to 3 PM for 10 days; n = 6.

(E) Binding of Clock to the mouse *Shp* promoter in *Clock*$^{mt/mt}$ and *Clock*$^{wt/wt}$ was studied by ChIP. Livers collected at different times were immunoprecipitated using anti-CLOCK antibodies and used to amplify E box sequences present in the *Shp* promoter. Representative electrophoresis image of the PCR products of clock binding (anti-Clock) and input is shown. This gel is a representative of n = 3.

(F–I) *Clock*$^{mt/mt}$ mice were injected with Ad-GFP or Ad-mShp (1×10^{11} virus particles/mouse). Plasma and liver were collected after 72 hr at 12:00 or 24:00 hr. Triglycerides were measured in total plasma and apoB-lipoproteins (F). Plasma was used to detect apolipoproteins by western blotting (G). Liver samples were used to measure Mtp mRNA and Gapdh mRNA (H) and Mtp protein (I). Each time point for mRNA represents mean ± SD; n = 3. *p < 0.05 compared to Ad-GFP at 12 hr.

(J) A schematic diagram suggesting that external stimuli such as food and light regulate cellular Clock. Clock regulates Shp, and Shp transmits this signal to Mtp. Changes in Mtp contribute to diurnal variations in plasma triglyceride.

that suppresses *MTTP*, then it should show circadian rhythm out of sync with MTP. Tissue analyses showed that hepatic *Shp* expression in C57BL/6J was high in the day compared to the night (Figures 6F and 6G). Note that MTP expression is low at this time (Figures 1E–1G and 6C–6E). *Shp* mRNA and protein showed rhythmic expression in the livers of *Clock*$^{wt/wt}$, but not in *Clock*$^{mt/mt}$ siblings (Figures 7A and 7B). The expression of Shp in *Clock*$^{mt/mt}$ mice was significantly low compared to that seen in their wild-type siblings (Figures 7A and 7B). Food entrainment studies revealed that Shp levels were reduced at the time of food availability in *Clock*$^{wt/wt}$, but not in *Clock*$^{mt/mt}$ mice (Figures 7C and 7D). These studies indicate that Shp shows daily variations, with peak expressions occurring in the day when Mtp expression is low. Food entrainment reduces Shp while enhancing Mtp expression. Therefore, Mtp and Shp show antiphasic expression profiles in wild-type mice fed *ad libitum* or subjected to food entrainment. However, similar to Mtp, Shp does not show daily variations in *Clock*$^{mt/mt}$ mice. In addition, we examined the association of Clock with the E box of Shp in vivo by ChIP. The binding of Clock to Shp E box sequences was high during 4 to 12 AM (Figure 7E), coincident with its high expression. In *Clock*$^{mt/mt}$, the binding of Clock to Shp promoter was low and did not show rhythmic change within 24 hr (Figure 7E). These data imply that the binding of Clock to Shp promoter is associated with increases in its expression.

Expression of SHP in Clock$^{mt/mt}$ Mice Abrogates Hypertriglyceridemia

The above studies indicated that Shp expression is low in *Clock*$^{mt/mt}$ and that it might contribute to high hepatic Mtp expression and hypertriglyceridemia. We then hypothesized that expression of SHP would decrease MTP expression and plasma triglyceride. Indeed, Shp expression significantly reduced triglyceride in plasma and apoB-lipoproteins (Figure 7F), plasma apoB100 and apoB48 (Figure 7G), and hepatic Mtp mRNA (Figure 7H) and protein (Figure 7I) without

affecting GAPDH expression (Figures 7H and 7I). These studies show that Shp decreases Mtp expression and ameliorates hypertriglyceridemia.

DISCUSSION

These studies provide evidence that Clock plays an important role in the circadian and food entrained regulation of hepatic *Mttp* expression and plasma triglyceride. We further show that CLOCK regulates MTP involving SHP (Figure 7J). CLOCK temporally interacts with the E box and increases *SHP* expression, whereas SHP reduces *MTTP* expression by differentially interacting with HNF4α and LRH-1. Decreased interaction of SHP with these transcription factors is associated with increased *MTTP* expression. Therefore, *Shp* is a clock-controlled gene that transmits information from *Clock* to *Mttp* (Figure 7J).

In $Clock^{mt/mt}$ mice, we found that smaller amounts of Clock bind to the *Shp* promoter, and this binding does not fluctuate within a day. Moreover, this binding leads to low *Shp* expression and consequently high *Mttp* expression at all times and sustained hypertriglyceridemia similar to that seen at night in $Clock^{wt/wt}$ mice. The major effect of the Clock mutant protein is that reductions in the Mttp expression seen in $Clock^{wt/wt}$ mice at dawn are absent in $Clock^{mt/mt}$ mice.

CLOCK and Other Circadian Genes in the Regulation of MTP and Plasma Triglyceride

$Clock^{mt/mt}$ mice and Huh-7 cells exposed to siCLOCK express more *MTTP*, indicating that $Clock^{mt/mt}$ mice have low Clock activity. These results are consistent with the observations that Clock plays an important role in the circadian regulation of liver and lung rhythms (DeBruyne et al., 2007). In addition to CLOCK, our cell culture studies indicate that BMAL1 and NPAS2 play similar roles in *MTTP* regulation, indicating that the positive loop of the circadian oscillators is important in reducing MTTP expression away from feeding times. In contrast, knockdown of the members of the negative circadian loop had no effect or reduced MTTP expression. It remains to be determined whether PERs and CRYs act directly on the *MTTP* promoter or reductions in Mttp expression after their knockdown is indirectly delivered via increased expression and activity of Clock/Bmal1.

Diurnal Regulation of SHP by CLOCK

SHP is a key transcriptional regulator of genes involved in diverse metabolic pathways and physiological functions (Chanda et al., 2008). Oiwa and associates observed rhythmic expression of Shp in the mouse liver (Oiwa et al., 2007) and demonstrated that Clock/Bmal1, along with Lrh-1, synergistically activate Shp promoter in vitro, and this activation is suppressed by Shp itself. They also showed that Clock binds to E box elements present in the Shp promoter. Here, we confirmed that Shp is rhythmically expressed in the liver (Figure 6). Moreover, we showed that the binding of Clock to the E box in the Shp promoter is high in the day. The rhythmic binding of Clock to the Shp promoter was synchronous with mRNA and protein accumulation during daytime in the mouse liver. In $Clock^{mt/mt}$ mice, however, the binding of Clock to Shp promoter did not show cyclic change, and Shp mRNA levels were relatively low (Figure 7). Similarly, levels of Per mRNA are low in $Clock^{mt/mt}$ (Figure S1). We propose that Shp is directly regulated by Clock, similar to Per proteins.

SHP expression did not change soon after serum supplementation in Huh-7 cells (Figure 5), indicating that it is not a serum-response gene. Later on, SHP displayed cyclic expression. The rhythmic expression of SHP was absent in siCLOCK-treated cells, pointing to a critical role of CLOCK. In siCLOCK-treated cells, low levels of CLOCK might not be sufficient to support cyclic induction of SHP transcription. This interpretation is consistent with an earlier conclusion that diurnal regulation of SHP is positively related with increased association of CLOCK with the SHP promoter. Although these studies indicate that changes in the association of CLOCK with SHP promoter regulate SHP expression, we did not observe comparable changes in CLOCK transcription, consistent with several studies (Mehra et al., 2009; Gallego and Virshup, 2007). Therefore, increased association of CLOCK with the SHP promoter is probably not a direct consequence of increased amounts of CLOCK. Instead, posttranslational modifications and its association with BMAL1 might determine enhanced association and subsequent dissociation of CLOCK from the SHP promoter (Mehra et al., 2009; Gallego and Virshup, 2007).

Shp is negatively regulated by Rev-erbα (Duez et al., 2008). Hence, we hypothesized that Rev-erbα would upregulate MTP expression. This is supported by several observations: siRev-erbα decreases Mtp expression in Huh-7 cells (Figure 3C and 3D); $Clock^{mt/mt}$ mice that have high MTP levels express low levels of Rev-erbα (Figures 1E–1G and S1); and neither Mtp nor Rev-erbα show temporal changes in $Clock^{mt/mt}$ mice (Figures 1E–1G and S1). Next, we theorized that, if Rev-erbα is directly involved in negative regulation of MTP, then their temporal expression would be anti-phasic, as we had seen between Mtp and Shp (Figure 7J). Instead, we found that both Rev-erbα and Mtp show peak expression at similar times. Therefore, it is likely that Rev-erbα indirectly regulates MTP via Shp. We propose that both the positive and negative loops of the circadian clock are involved in the diurnal regulation of MTP (Figure S5). Clock/Bmal1/Npas2 enhance Shp expression and reduce MTP expression in the daytime. High levels of Rev-erbα expression at night might suppress Shp expression and enhance Mtp expression.

Mechanisms Controlling Cyclic Expression of MTP in Liver Cells

Many studies have identified several transcription factors that regulate MTP expression (Hussain et al., 2008). Here, we recognized mechanisms that could contribute to rhythmic expression of MTP. First, our studies suggest that cyclic expression of *MTTP* involves differential association of HNF4α and HNF1α to its promoter. The bindings of these transcription factors to the promoter were high at the time of maximum *MTTP* expression. These transcription factors are known to synergistically activate *MTTP* expression (Sheena et al., 2005).

Second, binding of SHP to the *MTTP* promoter occurs in rhythmic fashion. At the time of high *MTTP* expression, lower amounts of SHP were found associated with HNF4α and LRH-1. In contrast, higher amounts of SHP were with the *MTTP* promoter, coincident with optimal SHP expression. Therefore, *MTTP* expression might be directly related to SHP levels. This was confirmed by overexpressing and reducing SHP. As discussed

above, Shp mRNA levels are high at the time of higher E box occupancy by the Clock in mice. Therefore, increased binding of Clock might lead to enhanced Shp expression. High Shp levels facilitate its association with MTP promoter causing repression.

Third, the rhythmic binding of HNF4α and HNF1α to the MTTP promoter was not seen in siCLOCK-treated cells (Figure 5H), indicating that CLOCK might also play a role in the association of HNF4α with the *MTTP* promoter. How CLOCK modulates the cyclic bindings of HNF4α and HNF1α to the *MTTP* promoter remains unknown. Under normal conditions, HNF4α expression shows no cyclic change (data not shown and Oiwa et al., 2007). Therefore, increased association of HNF4α with the *MTTP* promoter might involve posttranslational mechanisms such as differential binding of its ligands.

Regulation of Plasma Triglyceride

Metabolic syndrome and obesity are major metabolic disorders characterized by high plasma lipid concentrations. Plasma lipids are tightly controlled by mechanisms regulating their production and clearance. Here, we show that light-entrained mechanisms involving clock genes also play a role in regulating plasma triglyceride. We demonstrate that Clock regulates circadian expression of Mtp by controlling Shp in mice. Changes in Mtp are correlated with changes in plasma triglyceride, indicating that circadian changes in Mtp could contribute to changes in plasma apoB-lipoproteins and triglyceride. It is known that hepatic Mtp deficiency significantly lowers plasma triglyceride (Björkegren et al., 2002), and plasma triglycerides are very low in abetalipoproteinemia (Berriot-Varoqueaux et al., 2000). In $Clock^{mt/mt}$ mice, the amounts of plasma triglyceride were similar to those seen in $Clock^{wt/wt}$ siblings at night, suggesting that nadirs seen in wild-type mice at dawn are absent in these mice. Most likely, Clock plays a role in the nadirs seen in plasma triglyceride.

Triglyceride transfer activity of MTP has been exploited to identify several potent antagonists, which inhibit triglyceride transfer activity in vitro, suppress lipoprotein assembly and secretion in vivo, and decrease plasma lipids in humans and animals (Hussain et al., 2008). These drugs show several adverse effects. Dosing MTP inhibitors away from food intake diminishes intestinal distress. Based on the daily changes reported here for MTP, it might be interesting to evaluate the time of dosing on the pharmacokinetics of drug availability and efficacy, as well as in avoiding adverse effects.

In short, this study establishes a molecular link between circadian physiology and plasma lipid metabolism. These studies suggest that CLOCK regulates MTP expression using SHP. Although it is possible that other unidentified mechanisms might be involved, these studies point to the importance of both CLOCK and SHP in the circadian regulation of MTP and plasma triglyceride. Aberrations in the clock genes, as in $Clock^{mt/mt}$, lead to sustained hypertriglyceridemia because changes associated with dawn, such as upregulation of *Shp*, downregulation of *Mttp*, and reductions in plasma triglyceride, are absent.

EXPERIMENTAL PROCEDURES

Animals

Male 8- to 12-week-old C57BL/6J mice (Jackson Laboratory) were maintained in a 12 hr light/dark (LD) cycle (light on 7 AM to 7 PM) with free access to food and water. Heterozygous clock mutant breeding pairs (C57BL/6J-$Clock^{m1Jt}$/J, Jackson Laboratory, Stock No. 002923) were bred at Downstate Medical Center. $Shp^{+/+}$ and $Shp^{-/-}$ mice on a C57BL/6J background were maintained at the University of Utah. Male $Clock^{mt/mt}$ mice and their $Clock^{wt/wt}$ siblings, as well as $Shp^{+/+}$ and $Shp^{-/-}$ mice, were housed under a 12 hr light/dark cycle. Dissected tissues were quickly frozen and stored at –80°C. The Animal Care Committees at SUNY Downstate Medical Center and the University of Utah approved animal protocols.

Plasma Lipid Measurements

Total triglyceride and cholesterol were measured using kits (ThermoTrace Ltd). Lipids in high-density lipoproteins were measured after precipitating apoB lipoproteins. ApoB lipoprotein lipids were determined by subtracting HDL lipids from total lipid levels (Pan and Hussain, 2007).

Measuring MTP Activity

Livers (100 μg) from different mice were homogenized in 1 ml of buffer K (1.0 mM EGTA, 1 mM Tris-HCl, and mM $MgCl_2$) and centrifuged at 50,000 rpm for 1 hr. The supernatant was used to measure MTP activity using a kit (Chylos Inc.) as described (Athar et al., 2004).

RNA Extraction and Real-Time PCR Analysis

Total RNA was isolated from cells and tissues using Trizol reagent. Isolated RNA was reverse transcribed, and their levels were quantified by SYBR Green using an ABI Prism 7000 (Applied Biosystems). The primers used to measure different transcripts have been described (Pan et al., 2007; Pan and Hussain, 2007) and are shown in Table S1.

Small RNA Interference

siRNA directed against different clock genes and nonspecific control siRNA were obtained from Santa Cruz. They were introduced into Huh-7 cells plated in 12-well plates using siRNA transfection reagent (SC-29528, Santa Cruz). After 72 hr, cells were harvested for RNA or protein analysis.

Serum Shock

Huh-7 cells were grown to confluence in high-glucose Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS). Cells were starved in the same medium with no FBS for 18 hr, 50% horse serum was added for 2 hr, and then the medium was changed back to starvation medium (Balsalobre et al., 1998). Cells were harvested at 4 hr intervals for analyses.

Chromatin Immunoprecipitation Assay

Chromatin immunoprecipitation assay (ChIP) was performed according to the instructions (USB CHIP assay kit #78460) using goat polyclonal antibodies against CLOCK or SHP from Santa Cruz. DNA samples recovered after immunoprecipitation were subjected to PCR using primers previously described (Huang et al., 2007; Dai et al., 2010) and shown in Table S1. As negative controls, ChIP was performed in the absence of antibody or in the presence of rabbit IgG. These experiments were repeated three to four times with similar results. Data from a representative experiment are provided.

Plasmid Expression and Luciferase Assay

SHP expression using plasmids and adenoviruses has been described (Huang et al., 2007). Plasmid pMTP204 expressing a 204 kb *MTTP* promoter was described (Dai et al., 2010). Huh-7 cells were transfected with either the pMTP204 construct or empty vector along with a reporter *Renilla* luciferase construct and were assayed using the dual luciferase reporter assay system.

Statistical Analyses

Values are expressed as mean ± SD. One-way ANOVA was applied followed by Dunnett's two-tailed test or a t test. Cosinar analysis was performed using a program from http://www.circadian.org/softwar.html.

SUPPLEMENTAL INFORMATION

Supplemental Information includes five figures and one table and can be found with this article online at doi:10.1016/j.cmet.2010.05.014.

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